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The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response

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The discovery of a pharmacological treatment for phenylketonuria (PKU) raised new questions about function and dysfunction of phenylalanine hydroxylase (PAH), the enzyme deficient in this disease. To investigate the interdependence of the genotype, the metabolic state (phenylalanine substrate) and treatment (BH_4 cofactor) in the context of enzyme function *in vitro* and *in vivo*, we (i) used a fluorescence-based method for fast enzyme kinetic analyses at an expanded range of phenylalanine and BH_4 concentrations, (ii) depicted PAH function as activity landscapes, (iii) retraced the analyses in eukaryotic cells, and (iv) translated this into the human system by analyzing the outcome of oral BH_4 loading tests. PAH activity landscapes uncovered the optimal working range of recombinant wild-type PAH and provided new insights into PAH kinetics. They demonstrated how mutations might alter enzyme function in the space of varying substrate and cofactor concentrations. Experiments in eukaryotic cells revealed that the availability of the active PAH enzyme depends on the phenylalanine-to-BH_4 ratio. Finally, evaluation of data from BH_4 loading tests indicated that the patient's genotype influences the impact of the metabolic state on drug response. The results allowed for visualization and a better understanding of PAH function in the physiological and pathological state as well as in the therapeutic context of cofactor treatment. Moreover, our data underscore the need for more personalized procedures to safely identify and treat patients with BH_4-responsive PAH deficiency.

INTRODUCTION

Phenylketonuria (PKU; OMIM #261600), the most common inborn error of amino acid metabolism, is an autosomal recessive disorder caused by phenylalanine hydroxylase (PAH) deficiency (PAH; EC 1.14.16.1) (1). Currently, 627 different disease-causing mutations in the *PAH* gene are known (www.pahdb.mcgill.ca; www.hgmd.org) and some of these were shown to lead to protein misfolding with loss of function (2–4).

Pharmacological doses of 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4), the enzyme's natural cofactor, can reduce blood phenylalanine concentrations (5–10) and increase phenylalanine oxidation rates *in vivo* (11) in patients with PAH deficiency without any evidence of cofactor deficiency. The compound was shown to rescue the biochemical phenotype by correcting PAH misfolding and was thus classified as a pharmacological chaperone (4,12). Following these studies, sapropterin dihydrochloride, the synthetic form of the natural PAH cofactor, was approved as an orphan drug to alleviate or even replace burdensome dietary treatment in a significant share of patients with PKU due to PAH deficiency (13–16).

However, not all patients show BH_4 responsiveness. Since the introduction of sapropterin dihydrochloride as a pharmacological treatment, many attempts to predict BH_4 responsiveness from a patient's genotype were made (17–19).

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Combined evidence seems to support the view that residual enzyme activity of individual mutations may be a parameter that—to some extent—allows for discrimination of responders and non-responders (19–22). In two studies performed on Croatian and Turkish populations, calculation of mean *in vitro* residual enzyme activity of the two PAH variants arising from both alleles led to the identification of some responders with high accuracy, whereas patients with two fully inactive alleles were found to be always non-responders (19,22). Yet, in many instances, no clear genotype–phenotype correlation is found pointing to contributing factors such as the patient's age or initial blood phenylalanine concentrations (17,19). Marked inconsistencies as to BH_4 responsiveness were observed for two of the most common PKU mutations associated with this particular phenotype, R261Q and Y414C (17) and for R252W, L48S and R241C homozygous genotypes (22). In addition, interpretation of genotype effects is hampered by the fact that >80% of BH_4 responders are compound heterozygous (17).

Hydroxylation of the substrate L-phenylalanine to the product L-tyrosine with the use of the natural cofactor BH_4 and molecular oxygen is a complexly regulated catalytic mechanism. While L-phenylalanine induces activating conformational rearrangements, BH_4 leads to the formation of an inactive dead-end PAH–BH_4 complex (23–26). Recent studies unraveled new aspects concerning the interplay of phenylalanine and BH_4 having an impact on enzyme kinetics as well as on drug response. Adoption of an enzyme activity assay using a newly developed fluorescence-based real-time PAH activity assay revealed cooperativity of recombinant PAH towards the BH_4 cofactor. This was restricted to the phenylalanine substrate-activated state of the enzyme indicating that conformational rearrangements of the PAH protein induce cooperative binding (27). Moreover, investigations of the BH_4 effect in two different mouse models for BH_4-responsive PAH deficiency provided evidence that the response to BH_4 in terms of rescue of enzyme function by increasing the effective intracellular PAH amount also depends on phenylalanine concentrations and on the underlying genotype (12). These results suggested that the influence of substrate and cofactor concentrations in the presence of a certain genotype on enzyme function and on the response to the pharmacological chaperone BH_4 might be of even more functional and therapeutic relevance than previously estimated. In addition, the BH_4 loading test routinely used worldwide to assess BH_4 responsiveness in PAH deficiency (28,29) was shown to result in a number of inconsistencies that are still not well understood. In some but not all cases, this may be due to inadequate BH_4 dosage or to initial blood phenylalanine concentrations near to the physiological state. Unfortunately, this may lead to false negative results precluding cofactor treatment and thus increasing burden of treatment in some BH_4-responsive patients.

To address these issues, (i) we adapted our new fluorescence-based method for fast enzyme kinetic analyses to cover an expanded range of phenylalanine and BH_4 concentrations when compared with previous analytical setups enabling the investigation of the mutual impact of substrate and cofactor on PAH enzyme kinetics, (ii) we depicted these data as activity landscapes uncovering the optimal working range of recombinant wild-type and mutated PAH, (iii) we retraced these analyses in a eukaryotic cell culture system, revealing that the availability of the active PAH enzyme depends on both the metabolic state and drug dosage, and (iv) we translated this into the human system by analyzing the effect of the genotype, phenylalanine concentrations and the BH_4 dosage applied on the results of oral BH_4 loading tests from PAH-deficient patients.

RESULTS

Expanded insights into wild-type PAH kinetics unraveling the mutual impact of substrate and cofactor concentrations

In order to investigate the interdependence of L-phenylalanine and BH_4 in PAH enzyme kinetics, we adapted a newly developed fluorescence-based real-time activity assay (27) to simultaneously analyze the effect of a wide range of substrate and cofactor concentrations on PAH activity. Process automation now allowed for continuous measurement of tyrosine product formation over time in one single operation consisting of six sequential runs for all 96 wells (Fig. 1A). The assay was expanded to cover the space of 0 to 4000 μM L-phenylalanine and 0–500 μM BH_4 (Fig. 1B). First, we validated the data obtained with the new method by comparison with previous findings using a standard high-performance liquid chromatography (HPLC) based discontinuous assay (2). Data points determined at either varying L-phenylalanine concentrations (0–1000 μM) and one BH_4 concentration (75 μM) or at one L-phenylalanine concentration (1000 μM) and varying BH_4 concentrations (0–125 μM), respectively, were used to calculate enzyme kinetic parameters. Prior to calculation, an *F*-test was used to decide whether the Michaelis–Menten or the Hill kinetic model was more appropriate (27). Both L-phenylalanine and BH_4-dependent PAH enzyme kinetics showed clear data-fitting to the Hill equation (Fig. 1C), as previously described for the L-phenylalanine-activated enzyme (27). Although V_{max} values for L-phenylalanine and BH_4-dependent kinetics were higher in the new assay, allosteric parameters, i.e. apparent substrate affinity, the Hill coefficient and apparent cofactor affinity, were similar in both assays (Table 1).

We depicted the data analyzed by non-linear and polynomial regression fitting as three-dimensional landscapes of enzyme activity (30). This enabled a visual representation of the mutual impact of substrate (*x*-axis) and cofactor concentrations (*y*-axis) on PAH kinetics (color code) representing functional conditions of the PAH enzyme. Wild-type PAH showed a peak maximum enzyme activity at 575 μM L-phenylalanine and 125 μM BH_4, respectively (Fig. 1D). The analysis of PAH enzyme kinetics at BH_4 and L-phenylalanine concentrations extended to supraphysiological levels led to a number of interesting observations. High PAH enzyme activity was determined at a surprisingly wide range of substrate and cofactor concentrations. The optimal working range reflected by PAH enzyme activity in the boundaries of $[S]_{0.5}$ to K_i for the substrate and $[C]_{0.5}$ to K_i for the cofactor spanned from 252 to 2026 μM L-phenylalanine and from 44 to 306 μM BH_4 (Table 2). At L-phenylalanine concentrations above 561 μM, we observed the well-known substrate inhibition of enzyme activity (30,31). Notably, at cofactor

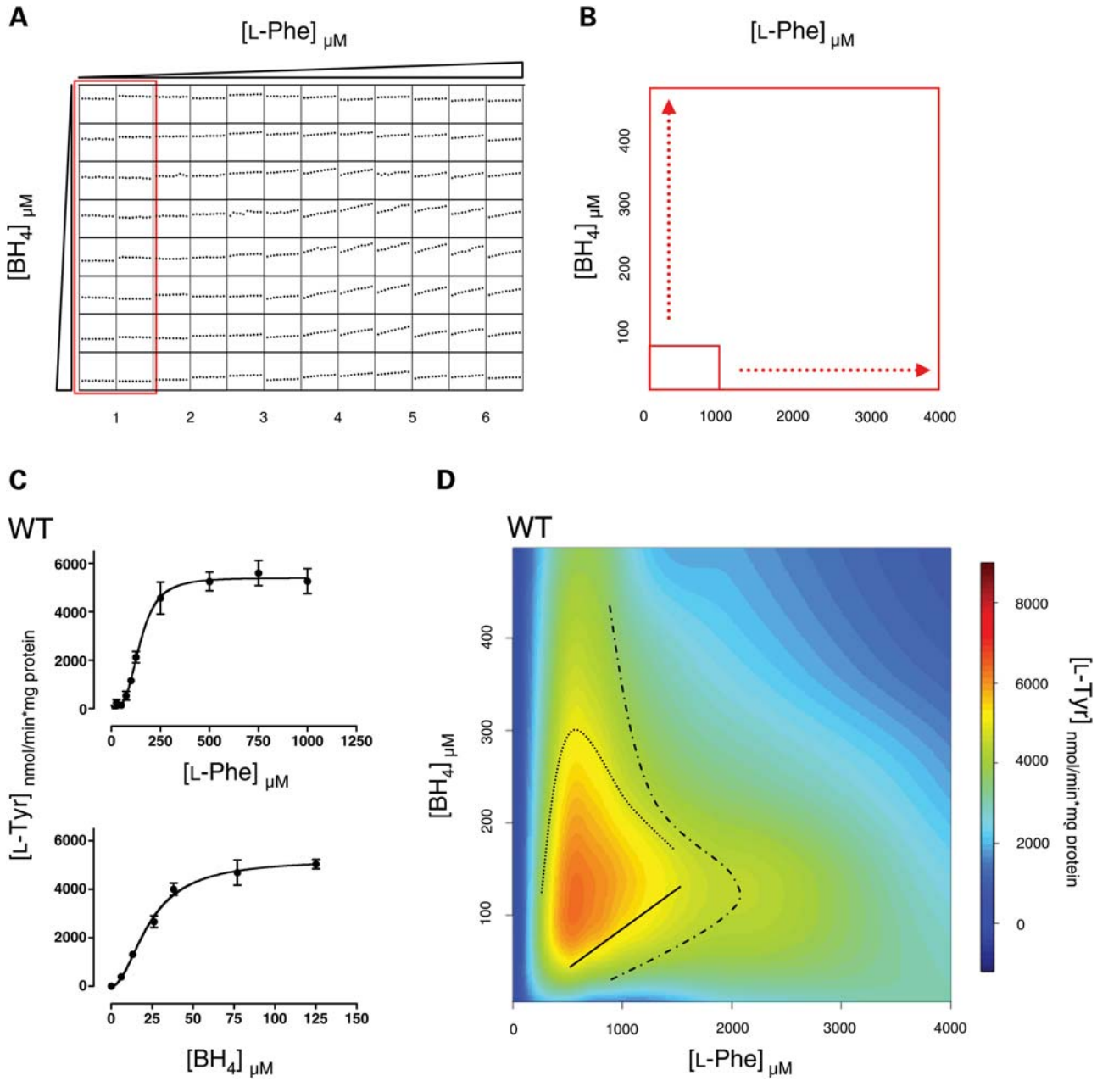

Figure 1. Optimal working range of wild-type PAH activity. (**A**) Scheme of sequential measurements of PAH enzyme kinetics in a 96-well plate. One sequence consists of two columns (red box). In each column, cofactor concentrations (0–500 µM) were varied at a fixed substrate concentration (0–4000 µM), respectively. Repeated cycles allowed for kinetic measurements of 16 wells over a time period of 90 s. (**B**) Extension of substrate and cofactor concentrations. The range of L-phenylalanine and BH_4 concentrations was expanded from standard conditions (BH_4, 75 µM; L-phenylalanine 1000 µM, red box) to 500 µM BH_4 and 4000 µM L-phenylalanine (arrows), respectively. (**C**) Cooperativity of PAH towards substrate and cofactor. Pre-activated PAH showed sigmoidal behavior for L-phenylalanine- (*upper panel*) and BH_4-dependent (*lower panel*) PAH enzyme kinetics. (**D**) Activity landscape of human wild-type PAH. Data for PAH enzyme activity assayed at varying L-phenylalanine and BH_4 concentrations were interpolated and depicted by a color code. The dot-and-dash line represents K_i for substrate inhibition at varying cofactor concentrations, the dotted line represents K_i for cofactor inhibition at varying substrate concentrations. With increasing substrate concentrations, more BH_4 is needed to maintain the same level of enzyme activity (solid line).

concentrations above 108 µM cofactor inhibition occurred. In addition, a mutual interdependence of both inhibitory events was found. These observations represent previously unknown findings. Over and above that, at L-phenylalanine concentrations within the range naturally occurring in the pathological state (500–1500 µM), the enzyme requires more BH_4 with increasing L-phenylalanine concentrations to maintain the same level of enzyme activity.

Taken together, the considerable extension of analysis conditions and the evaluation of data by compiling activity landscapes provided new insights into PAH kinetics. It allowed for a precise evaluation of peak PAH enzyme activity and the

Table 1. Comparison of enzyme kinetic parameters of human wild-type MBP–PAH at standard L-phenylalanine (1000 μM) and BH_4 (75 μM) concentrations

	L-phenylalanine[a]			BH_4[b]			
	V_{max} (nmol L-tyrosine/min × mg protein)	$[S]_{0.5}$ (μM)	h_{Phe}	V_{max} (nmol L-tyrosine/min × mg protein)	$[C]_{0.5}$ (μM)	K_m (μM)	h_{BH4}
Continuous assay	5407 ± 210	145 ± 11	3.3	5222 ± 286	23 ± 2		2.0
Discontinuous assay	3470 ± 75	155 ± 6	3.0	3425 ± 139		24 ± 3	

Steady-state kinetic parameters of wild-type MBP–PAH fusion proteins. V_{max} and the apparent affinities for L-phenylalanine ($S_{0.5}$) and BH_4 ($C_{0.5}$, K_m) as well as the Hill coefficient (h) as a measure of cooperativity are shown. Enzyme kinetic parameters were determined from enzyme activities measured using the newly developed fluorescence-based continuous assay and compared with enzyme activities measured by the standard HPLC-based discontinuous assay (2). Data were analyzed using the F-test for model comparison and the kinetic parameters of the best-fitting model (cooperative versus non-cooperative) were calculated. Values are given as mean ± SEM of three independent measurements.

[a]Enzyme kinetic parameters determined at variable L-phenylalanine concentrations (0–1000 μM) and standard BH_4 concentration (75 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

[b]Enzyme kinetic parameters determined at variable BH_4 concentrations (0–125 μM) and standard L-phenylalanine concentration (1000 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

Table 2. Peak enzyme activity and range of substrate and cofactor concentrations of wild-type and variant PAH

	Peak activity (nmol L-tyrosine/min × mg protein)	Residual activity (%)	L-phenylalanine concentration at peak activity (μM)	$[S]_{0.5}$–K_i[§]	BH_4 concentration at peak activity (μM)	$[C]_{0.5}$–K_i or K_m–K_i[§]
WT	6370	100	561	252–2026	108	44–306
F39L	5865	92	622	187–2275	143	35–331
I65T	3533	55	612	254–2075	135	29–322
R261Q	2654	42	842	344–2825	149	41–321
P275L	5215	82	1293	238–1980	334	71–435
P314S	1956	31	612	76–1043	80	42–218
V388M	6355	100	591	201–1933	105	47–296
Y414C[a]	3106	49	591	124–1048[a]	105	43–274
Y417H	5206	82	471	147–1501	92	63–262

Peak enzyme activity of variant tetrameric MBP–PAH fusion proteins measured by direct in-well activity measurements. The apparent affinities for L-phenylalanine ($S_{0.5}$) and BH_4 ($C_{0.5}/K_m$) as well as K_i for substrate and cofactor inhibition were calculated based on non-linear regression analysis at L-phenylalanine and BH_4 concentrations of peak enzyme activity.

[§]K_i calculated at peak L-phenylalanine and BH_4 concentrations using the Boltzman-sigmoidal equation.

[a]Calculation of K_i at a range of L-phenylalanine 0–1624 μM.

optimal working range spanning a wide area of substrate and cofactor concentrations. Moreover, cofactor inhibition as well as a mutual impact of substrate and cofactor concentrations on PAH activity have been identified.

Activity landscapes: the effect of *PAH* mutations on PAH enzyme kinetics

Variant PAH proteins harboring mutations mapping to the regulatory (F39L, I65T), the catalytic (R216Q, P275L, P314S, V388M) and the dimerization motif of the oligomerization domain (Y414C, Y417H) of the enzyme were analyzed. The results from kinetic measurements were depicted as activity landscapes and compared with those from wild-type PAH (Fig. 2). For each variant analyzed, enzyme kinetic parameters and peak enzyme activities with their corresponding L-phenylalanine or BH_4 concentrations were determined (Tables 2 and 3). The area of optimal enzyme activity defined as the range $[S]_{0.5}$ to K_i for the substrate and $[C]_{0.5}$ to K_i for the cofactor, respectively, was calculated (Table 2).

The variant proteins bearing the mutation F39L or I65T, both located in the regulatory domain, showed at first glance a landscape pattern comparable with that of the wild-type. Besides enzyme activity (F39L, 92%; I65T, 55%), the variants displayed unaffected enzyme kinetic parameters as well as similar effects of substrate and cofactor inhibition as found for the wild-type enzyme. However, a more detailed analysis revealed that peak PAH activities were found at similar substrate concentrations, but at ~1.3-fold higher cofactor concentrations when compared with the wild-type. This indicates that in the presence of these mutations, more cofactor is needed to achieve optimal PAH function.

All mutations mapping to the catalytic domain induced marked alterations of activity landscapes. For the variant R261Q, the area of substantial PAH activity was much larger than for the wild-type, but residual enzyme activity was reduced to 42%. The concentrations of substrate and cofactor needed to achieve peak PAH activity were shifted to higher values (L-phenylalanine, 1.5-fold; BH_4, 1.4-fold). We observed reduced affinity to the substrate by a factor of 0.5 ($[S]_{0.5}$ 282 μM). Binding of BH_4 followed Michaelis–Menten kinetics representing a loss of cooperativity that occurred with reduced affinity (K_m 66 μM). Broadening of the landscape resulted from a significant shift of enzyme inhibition. Notably, the variant did not display cooperative binding of BH_4 when activated. Taken together, the unique feature of

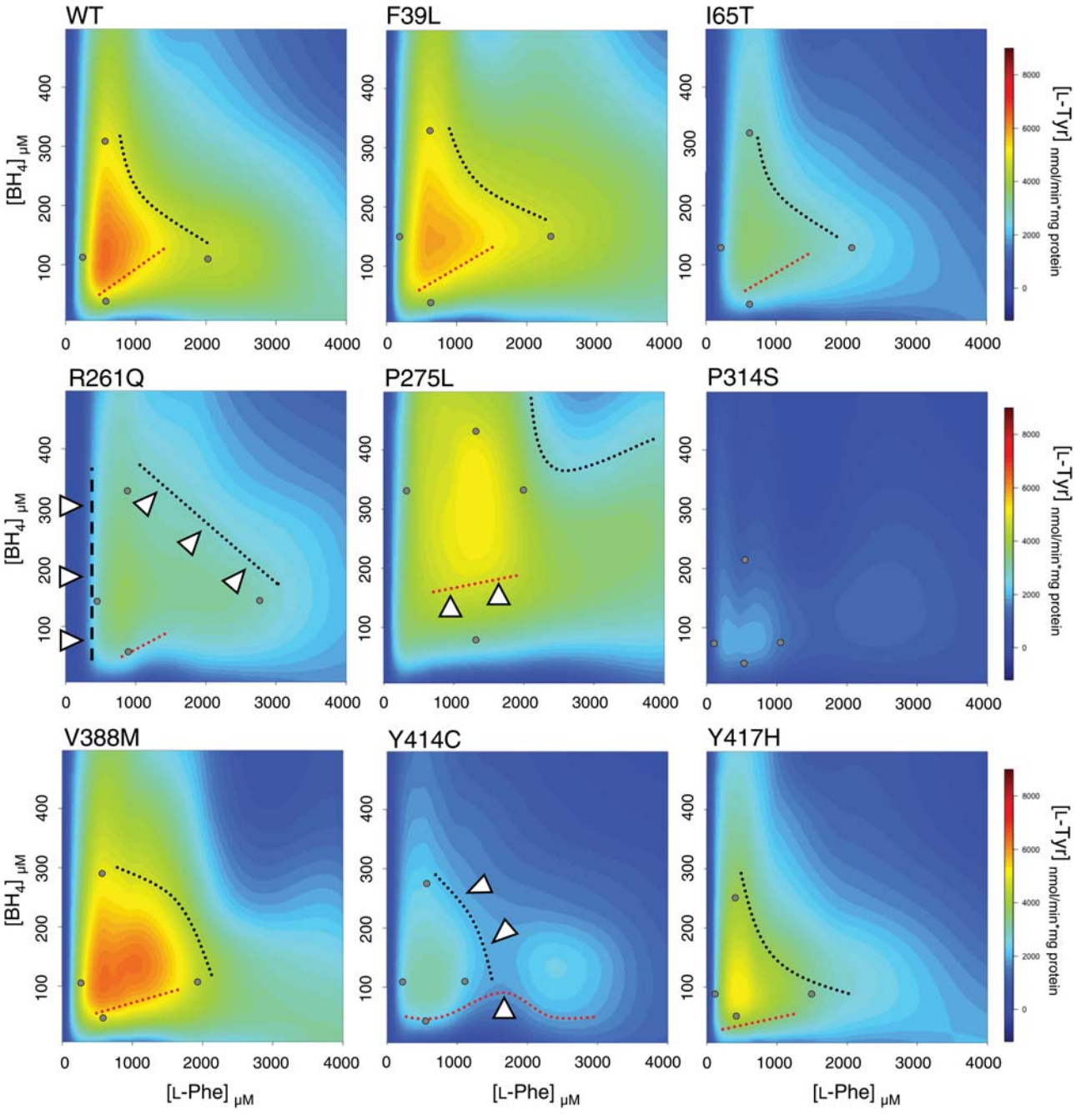

Figure 2. Activity landscapes of recombinant wild-type and variant PAH. The interpolated specific enzyme activities are color-coded and given as a function of different L-phenylalanine and BH_4 concentrations. Substrate and cofactor inhibition are depicted as a summation line (black dotted line). Positions of $S_{0.5}$ and K_i for L-phenylalanine and $C_{0.5}$ and K_i for BH_4, respectively, are indicated at peak enzyme activities (open circles). Marked changes in the activity landscape of variant PAH when compared with the wild-type (WT) are highlighted (open triangles) and BH_4 concentrations needed to maintain the same enzyme activity with increasing L-phenylalanine concentrations are shown (red dotted line).

the R261Q variant was the right shift of PAH enzyme activity towards higher L-phenylalanine concentrations indicating a reduced affinity of this variant to its substrate and that the enzyme displays low activity at L-phenylalanine concentrations below 600 µmol/l. At these substrate concentrations, even very high BH_4 doses would not produce any response in PAH activity. The presence of the mutation P275L resulted in an enzyme with overall high residual enzyme activity (82%), yet with a shift of peak enzyme activity to 2.3-fold higher L-phenylalanine and 3-fold higher BH_4 concentrations (L-phenylalanine 1293 µM, BH_4 334 µM) than the wild-type. In comparison to the wild-type, the enzyme had a substantially higher need for BH_4 to achieve the optimal working range. As a consequence, substrate and cofactor inhibition was almost abolished. The variant protein bearing the mutation P314S showed a severe loss in residual enzyme activity (31%) with

Table 3. Enzyme kinetic parameters at standard L-phenylalanine (1000 μM) and BH_4 (75 μM) concentrations calculated from activity landscapes

	L-phenylalanine[a]			BH_4[b]			
	V_{max} (nmol L-tyrosine/min × mg protein)	$[S]_{0.5}$ (μM)	h_{Phe}	V_{max} (nmol L-tyrosine/min × mg protein)	$[C]_{0.5}$ (μM)	K_m (μM)	h_{BH4}
WT	5407 ± 210	145 ± 11	3.3	5222 ± 286	23 ± 2		2.0
F39L	4961 ± 342	115 ± 15	2.7	5669 ± 311	36 ± 3		1.7
I65T	3166 ± 96	161 ± 10	2.7	3277 ± 196	27 ± 3		1.7
R261Q	3041 ± 128	282 ± 18	3.4	4693 ± 598		66 ± 17	
P275L	3204 ± 126	112 ± 8	2.6	3022 ± 179	36 ± 3		2.8
P314S	1650 ± 91	76 ± 9	1.8	991 ± 25	24 ± 1		5.2
V388M	5639 ± 112	140 ± 6	2.7	5895 ± 236	26 ± 2		1.8
Y414C	2895 ± 206	120 ± 17	2.4	2148 ± 203	28 ± 4		2.6
Y417H	4434 ± 247	111 ± 10	3.6	3011 ± 93	23 ± 1		2.5

Steady-state kinetic parameters of variant tetrameric MBP–PAH fusion proteins determined by direct in-well activity measurements. Data were analyzed using the *F*-test for model comparison and the kinetic parameters of the best-fitting model (cooperative versus non-cooperative) were calculated. V_{max} and the apparent affinities for L-phenylalanine ($S_{0.5}$) and BH_4 ($C_{0.5}$, K_m) as well as the Hill coefficient (h) as a measure of cooperativity are shown. Values are given as mean ± SEM of three independent measurements.

[a]Enzyme kinetic parameters determined at variable L-phenylalanine concentrations (0–1000 μM) and standard BH_4 concentration (75 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

[b]Enzyme kinetic parameters determined at variable BH_4 concentrations (0–125 μM) and standard L-phenylalanine concentration (1000 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

a narrow optimal working range that was shifted towards lower L-phenylalanine as well as BH_4 concentrations. In addition, enzyme kinetics revealed a significantly increased apparent affinity to the substrate with marked reduction in cooperativity. In contrast to previous findings (3,32), where the variant protein V388M was described as a K_m variant with reduced affinity to the cofactor, we detected high residual enzyme activity over an expanded range of substrate and cofactor concentrations. Residual PAH activity was 100% and peak catalysis (L-phenylalanine 591 μM, BH_4 105 μM) as well as effects of substrate and cofactor inhibition were similar to that of the wild-type enzyme.

Mutations mapping to the oligomerization domain, Y414C and Y417H, showed a narrowed optimal working range that was shifted to lower substrate concentrations. However, this was less pronounced for the milder mutation Y417H (82% residual enzyme activity) when compared with Y414C (49%). On the other hand, Y417H needed less BH_4 to achieve the area of optimal function. Interestingly, different to all variants analyzed, Y414C showed two peaks of high enzyme activity.

Taken together, we identified many similarities between the activity landscapes of the wild-type and variant PAH proteins, showing generally high residual enzyme activity as well as substrate and cofactor inhibition. However, these activity landscapes also revealed important differences in the regulation of PAH activity by BH_4 and L-phenylalanine and helped to visualize the interdependence of substrate and cofactor concentrations on variant PAH enzyme activity.

Cofactor action on PAH function in respect of kinetic behavior and the chaperone effect

We used two different approaches to analyze different aspects of cofactor action on the wild-type, R261Q and Y414C PAH enzyme in HEK293 cells. First, we in part retraced the activity landscapes analyzing the kinetic behavior by assaying enzyme activity of cell lysates at different L-phenylalanine concentrations (0–1000 μM) while keeping the BH_4 concentration constant (75 μM) (Fig. 3A). Secondly, we analyzed the chaperone effect of the BH_4 cofactor at different phenylalanine and BH_4 concentrations after a 72 h incubation.

As expected, the wild-type protein showed the highest enzyme activity of all three proteins analyzed with a peak activity at about 250 μM L-phenylalanine. The latter finding differed from the peak activity observed for recombinant PAH at ~500 μM. As seen for prokaryotic PAH, a further increase in L-phenylalanine concentrations resulted in substrate inhibition. The variant R261Q displayed ~50% residual enzyme activity and a lower slope of the curve that leveled off at a plateau. Reduced enzyme function, which was shifted toward higher substrate concentrations with a broadened working range, substantiated results from activity landscapes. Residual enzyme activity of eukaryotic Y414C was substantially lower than the specific activity of the corresponding recombinant variant. In addition to peak activity at 180 μM L-phenylalanine, a second slight incline of activity was observed at high L-phenylalanine concentrations (1000 μM). Although generally shifted toward lower substrate concentrations, this is in line with the two peaks observed in the activity landscape.

Next, we aimed to elucidate the long-term chaperone effect of varying intracellular phenylalanine and BH_4 concentrations on PAH function. The determination of enzyme activity at standard conditions after previous incubation of cells with different substrate and cofactor concentrations assays the availability of functionally active PAH. For this purpose, stably transfected cells were cultivated with L-phenylalanine concentrations of 91, 500 or 1200 μM and BH_4 concentrations of 0, 40 or 75 μM for 72 h. First, cells were cultivated at L-phenylalanine levels representing the physiological state (91 μM), mild PKU (500 μM) or classical PKU (1200 μM) without the addition of BH_4 to the medium (Fig. 3B). Wild-type PAH activity showed a trend towards an increase only at clearly pathological L-phenylalanine concentrations. For R261Q, a steady but statistically not significant increase in enzyme activity was seen

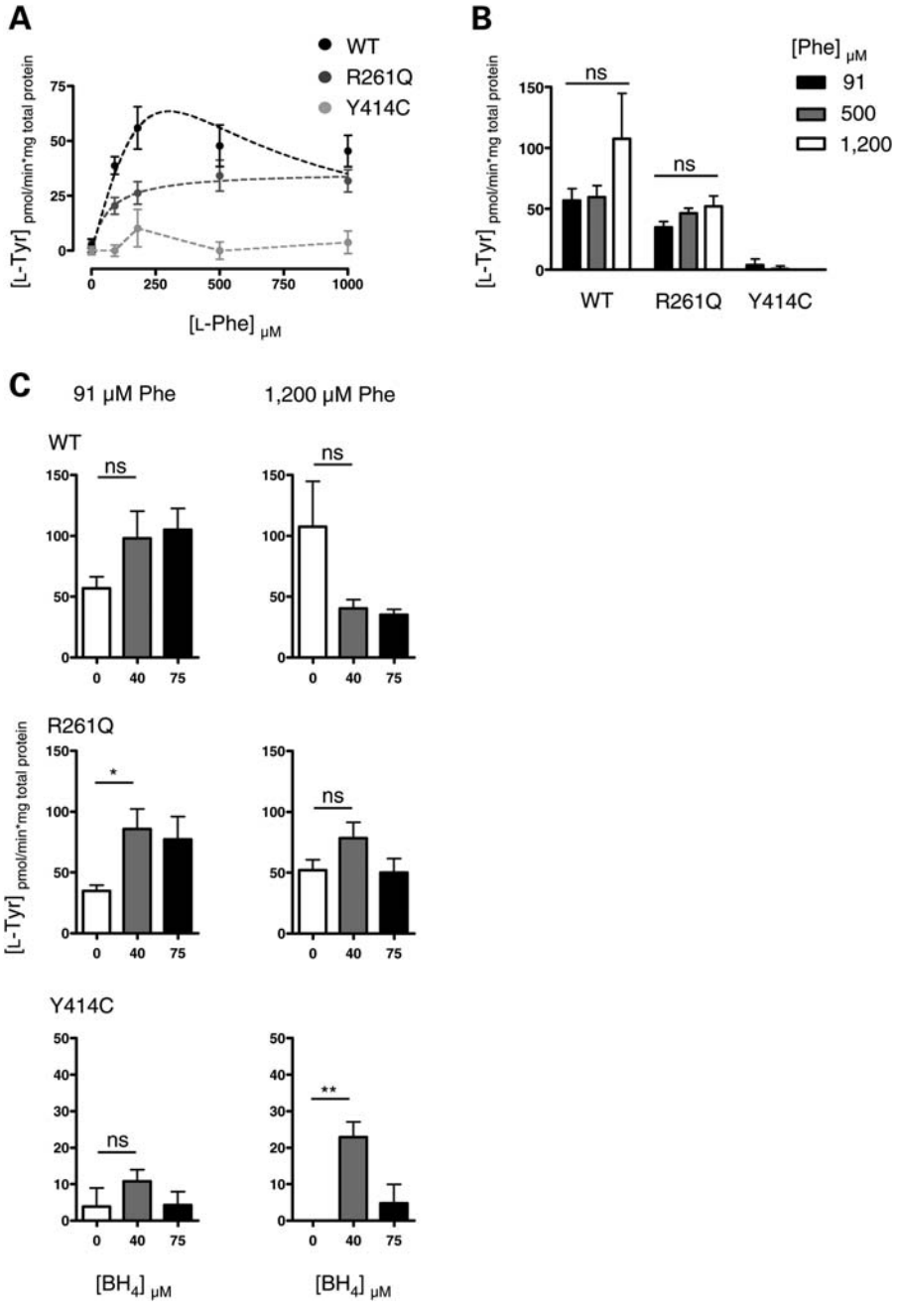

Figure 3. Cofactor action on PAH function in respect of kinetic behavior and the chaperone effect. (**A**) Enzyme activity of eukaryotic expressed PAH assayed at varying substrate concentrations. Enzyme activities in cell lysates of HEK293 cells stably transfected with wild-type PAH, R261Q or Y414C were determined at varying L-phenylalanine concentrations (0–1000 µM) and a fixed BH_4 concentration (75 µM). (**B**) Effect of varying intracellular phenylalanine concentrations on functionally active PAH. PAH enzyme activity in HEK293 cell lysates expressing wild-type PAH, R261Q or Y414C was determined at standard assay concentrations (1000 µM L-phenylalanine and 75 µM BH_4) after prior incubation with different L-phenylalanine concentrations (black, 91 µM; dark gray, 500 µM; white, 1200 µM) for 72 h. (**C**) Mutual impact of varying substrate and cofactor concentrations on functionally active PAH. Cells stably expressing wild-type PAH, R261Q or Y414C were cultivated at different phenylalanine (left column, 91 µM; right column, 1200 µM) and BH_4 concentrations (white, 0; dark gray, 40 µM; and black, 75 µM). PAH enzyme activities were analyzed at standard assay conditions. PAH activities are given as mean ± SEM of three independent experiments. Significant differences in enzyme activities were calculated using one-way ANOVA and Dunnett's multiple comparison test (ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$).

upon increasing substrate concentrations in the medium (35 ± 5 to 52 ± 9 pmol L-tyrosine/min × mg total protein). For Y414C, residual activity was very low without any effect of increasing L-phenylalanine concentrations.

To further evaluate the mutual impact of the substrate and the cofactor on enzyme activity in the eukaryotic system, 40 or 75 µM BH_4 were added to the medium at L-phenylalanine concentrations of 91 or 1200 µM, respectively (Fig. 3C).

Interestingly, at physiological L-phenylalanine concentrations (91 μM), the addition of 40 and 75 μM BH_4 induced a trend towards increased enzyme activity of wild-type PAH (57 ± 9 to 98 ± 22 and 105 ± 18 pmol L-tyrosine/min × mg total protein), whereas the opposite effect was observed at elevated L-phenylalanine concentrations (1200 μM L-phenylalanine, 108 ± 37 to 40 ± 7 and 35 ± 5 pmol L-tyrosine/min × mg total protein). For the variant R261Q, the addition of 40 μM BH_4 at physiological L-phenylalanine concentrations resulted in a significant increase in enzyme activity (35 ± 5 to 86 ± 17 pmol L-tyrosine/min × mg total protein), but the inhibitory effect at elevated L-phenylalanine concentrations as seen for the wild-type was not observed. At physiological L-phenylalanine concentrations, the variant Y414C showed low residual enzyme activity with only a minor increase upon the addition of 40 μM BH_4. Interestingly, at high L-phenylalanine concentrations (1200 μM), the addition of 40 but not 75 μM BH_4 led to a significant increase in residual enzyme activity (23 ± 4 pmol L-tyrosine/min × mg total protein) achieving as much as 40% of the wild-type level.

Taken together, findings from the prokaryotic system depicted by activity landscapes were substantiated in the eukaryotic environment. Also in this system probing lysates of cultured cells, the mutual impact of different L-phenylalanine and BH_4 concentrations on enzyme activity varied among different PAH variants with substrate inhibition for the wild-type as well as constant activity levels for R261Q also at very high L-phenylalanine concentrations. Residual enzyme activity of Y414C was substantially lower in cell culture when compared with the specific activity of the recombinant protein, but enzyme function was rescued by the addition of BH_4. This may point to impaired protein stability in the eukaryotic environment and a stabilizing pharmacological chaperone effect by BH_4.

The mutual impact of substrate and cofactor concentrations on results from BH_4 loading tests performed in PAH deficient patients

So far, data pointed to a simultaneous dependency of PAH function from available substrate and cofactor concentrations. After having confirmed results from the prokaryotic system in the eukaryotic system, we aimed to investigate whether our observations may be transferred to the human situation by analyzing data from BH_4-loading tests performed in patients with PAH deficiency. To address this issue, we collected data of patients homozygous or functionally hemizygous for the mutations F39L, I65T, R261Q or Y414C, that underwent a BH_4-loading test with a dose of 20 mg/kg body weight and a duration of at least 24 h from the literature (19,33–37) and from the BIOPKU database (www.biopku.org). First, we compiled the course of blood phenylalanine values within 24 h after a single dose of BH_4 as a function of initial blood phenylalanine concentrations. For all mutations analyzed, different blood phenylalanine concentrations at the beginning of the test led to differences in the extent of BH_4 responsiveness, i.e. the percent decrease in blood phenylalanine after drug administration. Patients carrying the mutations F39L, I65T and Y414C showed a peak level of BH_4 responsiveness below an initial blood phenylalanine concentration of 500 μM (Fig. 4A). In the presence of F39L and I65T increasing initial blood phenylalanine concentrations were associated with a decrease in BH_4 responsiveness. While patients with F39L still displayed positive response at 1000 μM phenylalanine (>30% decrease in blood phenylalanine), those carrying I65T did not show a drug response when initial blood phenylalanine concentrations were >800 μM. The mutation Y414C led to inconsistent response to BH_4 with maximum responsiveness at initial blood phenylalanine concentrations up to 750 μM phenylalanine and at 1000 μM phenylalanine. In general, patients bearing the mutation Y414C showed a high degree in BH_4 responsiveness with the lowest response remaining within the range of 30% decrease in blood phenylalanine.

Patients bearing the mutation R261Q showed strong inconsistencies in BH_4 responsiveness with some patients displaying high levels of responsiveness (>60% decrease of blood phenylalanine) and others no response at all (Fig. 4B). To investigate the basis of these inconsistencies, we analyzed the impact of both the genotype on BH_4 response and of phenylalanine concentrations on different genotypes comprising the R261Q mutation. The decrease in blood phenylalanine concentrations was significantly stronger in individuals carrying the R261Q mutation in the homozygous state (median, 37.5%) than those carrying it in the functional hemizygous state (median, 6.5%). However, among homozygous patients, some were responders and some were not, whereas all functionally hemizygous patients had to be classified as non-responders. Interestingly, the level of initial blood phenylalanine did not allow differentiating either homozygous responders from homozygous non-responders or homozygotes from functional hemizygotes.

In addition to the influence of initial blood phenylalanine concentrations on BH_4 response, we analyzed the impact of the genotype on BH_4 dose response to investigate the optimal PAH working range *in vivo*. Literature data providing information on BH_4 dosage revealed clear differences for patients homozygous for R261Q, patients homozygous for Y414C and those compound heterozygous for these two mutations (Fig. 4C). In the presence of the mutation R261Q, the percent decrease in blood phenylalanine levels remained nearly unchanged for the range of BH_4 dosages between 10 and 30 mg/kg body weight. In contrast, patients bearing the mutation Y414C showed a gain in BH_4 response with increasing BH_4 doses (5–20 mg/kg body weight). Interestingly, patients compound heterozygous for R261Q and Y414C showed an intermediate response with respect to the BH_4 dosage administered (10–20 mg/kg body weight) when compared with patients homozygous for these mutations.

As a conclusion, *in vivo* PAH activity is a function of the phenylalanine substrate and the BH_4 cofactor as well as the patient's genotype. Hence, enzyme function in the individual patient at a given time point is the resultant of the metabolic state and the dosage of cofactor treatment both in turn determined by the underlying mutations.

DISCUSSION

Regulation of PAH activity is essentially governed by the abundance of the phenylalanine substrate and the BH_4

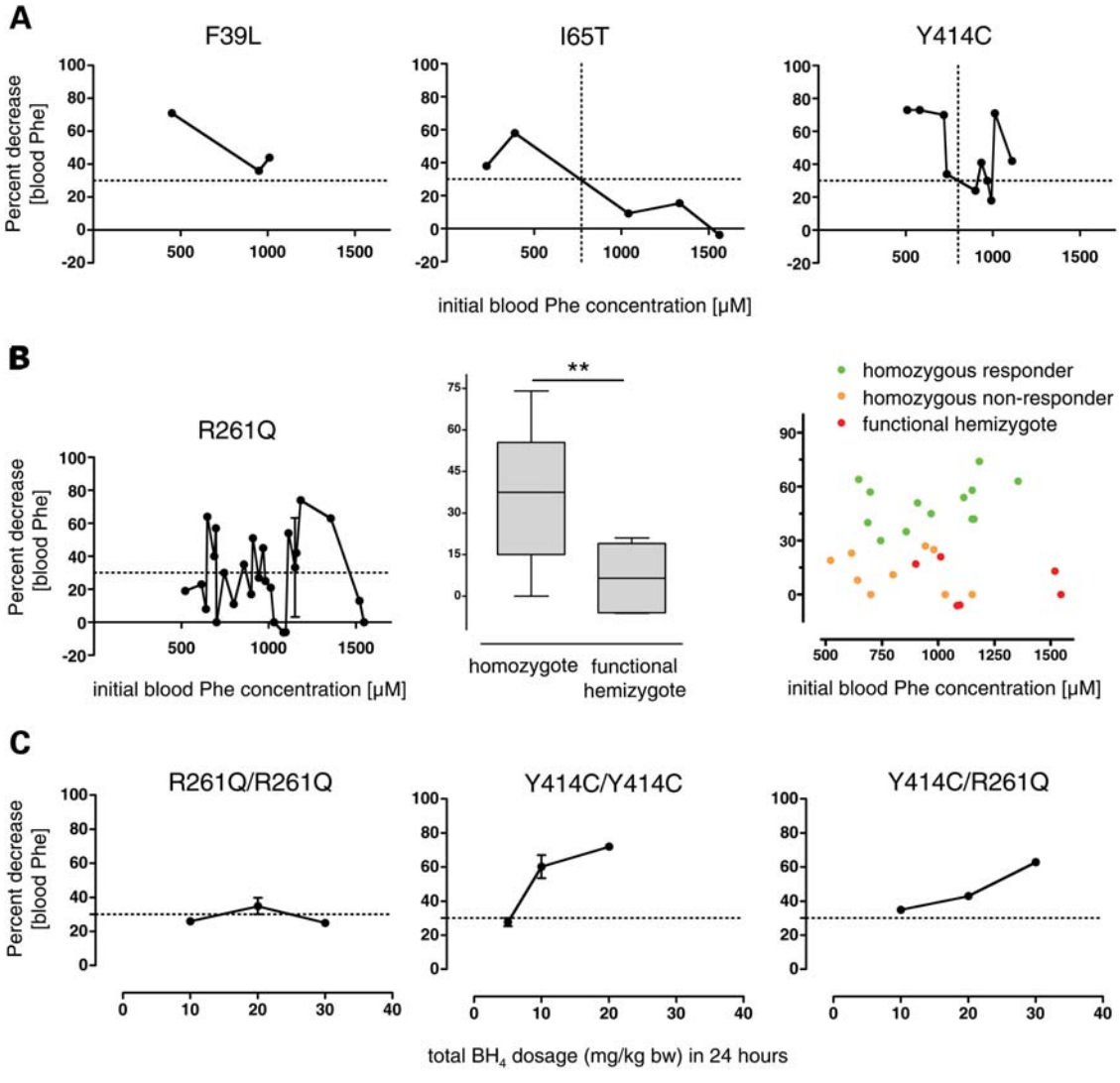

Figure 4. Evaluation of BH_4 responsiveness in PAH-deficient patients. (**A**) The impact of the genotype and of initial blood phenylalanine concentrations on BH_4 response. The graphs show the percent decrease in blood phenylalanine levels 24 h after a single dose of BH_4 (20 mg/kg bw) in patients homozygous or functional hemizygous for the mutations F39L ($n = 3$), I65T ($n = 5$) or Y414C ($n = 10$) as a function of different blood phenylalanine values at the beginning of the test. The horizontal dashed line indicates 30% decrease in blood phenylalanine concentrations, the arbitrary criterion of BH_4 responsiveness. The vertical dashed line shows the initial blood phenylalanine concentration, above which BH_4 leads to a blood phenylalanine decrease of $<30\%$. (**B**) BH_4 responsiveness in patients carrying the mutation R261Q ($n = 28$). (Left) Percent decrease in blood phenylalanine 24 h after a single dose of BH_4 (20 mg/kg bw) as a function of initial blood phenylalanine values in patients homozygous or functional hemizygous for the R261Q mutation. (Middle) Percent decrease in blood phenylalanine 24 h after a single dose of BH_4 (20 mg/kg bw) in patients carrying the R261Q mutation in the homozygous state ($n = 22$) in comparison to functional hemizygotes ($n = 6$). The boxes show the interquartile ranges (25th to 75th percentiles), the horizontal black bars represent the median, the bars indicate the range. Significance is indicated ($^{**}P < 0.01$, unpaired Student's *t*-test). (Right) Percent decrease in blood phenylalanine concentrations in function of initial blood phenylalanine values in homozygous responders ($n = 13$) and non-responders ($n = 9$) as well as functional hemizygous patients ($n = 6$) displaying the mutation R261Q. (**C**) The impact of the genotype on BH_4 dose response. Percent decrease in blood phenylalanine 24 h after a single application of BH_4 in different dosages in patients homozygous for R261Q ($n = 24$) (*left*), homozygous for Y414C ($n = 10$) (*middle*) or compound heterozygous for R261Q/Y414C ($n = 3$) (*right*).

cofactor. The supply of substrate, e.g. upon food intake, induces enzyme activation and subsequently full catalytic activity. In contrast, at low blood phenylalanine levels, e.g. under fasting conditions, BH_4-induced PAH inhibition prevents from undue elimination of the essential amino acid phenylalanine. Thus, it is the ratio of phenylalanine to BH_4 that determines activation and inhibition of the enzyme. However, the concentration of BH_4 in the liver cell is held rather constant, whereas phenylalanine levels undergo substantial fluctuations in function of the metabolic state and—in the presence of PAH deficiency—of the underlying genotype. While treating patients with BH_4 we intervene in this system without disposing of profound knowledge concerning the effect of shifts of the substrate-to-cofactor ratio. In the work presented here, we first wanted to apply the newly developed real-time fluorescence-based PAH enzyme activity assay to identify the optimal working range of the enzyme with respect to these substances. Secondly, we aimed to analyze

the influence of changes in the phenylalanine-to-BH_4 ratio on PAH function of the wild-type and variant enzymes.

Analyses of wild-type PAH revealed two interesting new findings. In addition to the well-known enzyme inhibition by the substrate, cofactor inhibition was identified by the extension of the enzyme activity assay to much higher cofactor concentrations. Moreover, we learned that with increasing phenylalanine concentrations, more BH_4 is needed to maintain the same level of PAH activity. Still, it has to be considered that the full range of substrate and cofactor concentrations applied in our novel assay provided new insights into theoretical aspects of PAH enzymology, but it is well beyond physiological levels. However, our approach allowed for detailed visualization and better understanding of conditions corresponding to those occurring at the edges of classical pathological situations in PKU patients and in the therapeutic context upon cofactor treatment.

The optimal working range of the wild-type enzyme occurred at phenylalanine concentrations of 250–500 μM. From a physiological point of view, this appears reasonable, since upon food intake liver phenylalanine concentrations are expected to reach levels up to 500 μM rather than around 1000 μM, the phenylalanine concentration at which standard PAH enzyme activity assays are performed (2,38). Our results showed that the optimal BH_4 concentration for PAH enzyme activity is ∼100 μM, while standard PAH activity assays are run at 75 μM BH_4 (2,38). In any case, the physiological cellular BH_4 concentration in the liver is by far lower (∼8.5 μM) (26), implying that the cell has always to be considered BH_4 deficient in view of the enzymatic task. Yet, in light of the inhibitory potential of BH_4 on the enzyme, a cellular cofactor concentration significantly below the K_m value of 23 μM reduces inhibition when activity is needed, i.e. at phenylalanine concentrations above the physiological range. The trade-off between these two tasks is balanced by a 6-fold higher affinity of the enzyme toward the cofactor (K_m, 23 μM) than toward the substrate ($S_{0.5}$, 145 μM). As a consequence, PAH binds BH_4 and phenylalanine at a ratio of 0.5 at 100 μM blood phenylalanine (inhibition) when compared with 0.09 at 561 μM blood phenylalanine (no inhibition). These theoretical assumptions are corroborated by previous *in vivo* ^{13}C-phenylalanine oxidation tests performed in a PKU mouse model (39). In the euphenylalaninemic state, a hypothetical BH_4 deficiency is overruled by an inhibitory effect following the application of BH_4 in wild-type animals. However, in hyperphenylalaninemia phenotypes, the administration of BH_4 leads to an immediate increase in ^{13}C-phenylalanine oxidation. In view of a time to effect of $<$5 min, this has to be considered independent of a pharmacological chaperone effect and rather points to compensation of BH_4 deficiency in this metabolic state. Comparable studies in humans addressing phenylalanine concentrations, BH_4 concentrations, the effect of BH_4 in healthy individuals, time to onset of action and effect duration would be of interest, but have not yet been performed.

Next, we investigated the effect of selected missense mutations in the *PAH* gene on the optimal working range of the enzyme. In general, activity landscapes of the wild-type and variant PAH proteins displayed comparable patterns with rather high residual enzyme activity and a limited area of maximum activity. More detailed analyses, however, revealed various alterations with respect to the extension and position of the optimal working range in the coordinates of substrate and cofactor concentrations. Most variant proteins were in need of more BH_4 to achieve peak activity (F39L, I65T, R261Q, P275L, Y417H, Y414C). For two variants (R261Q and P275L), maximum activity was determined at markedly higher phenylalanine concentrations (842 and 1293 μM). In contrast, three variants (P314S, Y414C and Y417H) presented a narrowed optimal working range that was shifted to lower phenylalanine concentrations when compared with the wild-type.

Two of these mutations, R261Q and Y414C, are frequent, but inconsistently associated with BH_4 responsiveness (17,34–37). The analysis of activity landscapes provided first evidence for an impact of the metabolic state on variant PAH function. R261Q displayed marked reduction in enzyme activity at phenylalanine concentrations in the therapeutic range below 240 μM giving rise to the hypothesis that patients bearing this mutation in the homozygous or functional hemizygous state would not benefit from a restrictive dietary regime. On the other hand, the mutation Y414C that induces a shift of activity to lower phenylalanine concentrations would require a rather strict metabolic adjustment with low blood phenylalanine values to achieve optimized PAH activity. Thus, observations from the analysis of activity landscapes could be of importance for a deeper understanding of inconsistent results from BH_4 loading tests or some disappointing experiences upon BH_4 treatment of our patients. To perform a further step in this direction, we carried out cell culture experiments and analyzed data from BH_4 loading tests performed in PKU patients.

Data extracted from activity landscapes were reproduced in the setting of stably transfected cells. Having gained insights into the mutual impact of varying substrate and cofactor concentrations on PAH activity, we were then interested in answering the question of how changes in the metabolic state would affect the effective PAH concentration, that is, the intracellular amount of functional PAH enzyme available for phenylalanine conversion. Since BH_4 has been classified as a pharmacological chaperone, i.e. a stabilizing compound that helps to overcome PAH degradation in the cell, it is expected to raise the amount of PAH in cell culture. In addition, we had previously shown that the effective PAH concentration is influenced by changes in the phenylalanine-to-BH_4 ratio in the mouse (12). To address these issues in cell culture, we mimicked physiological (euphenylalaninemic) and pathologic (hyperphenylalaninemic) conditions representing classical PKU. The supplementation of BH_4 induced diverse results in the presence of R261Q and Y414C, respectively. BH_4 was beneficial for catalytic PAH function of the R261Q variant particularly at low phenylalanine levels, whereas a significant increase in PAH enzyme activity reflecting an increase in the effective PAH amount was observed for Y414C under PKU conditions. Interestingly, the therapeutic range for BH_4 was narrow for Y414C, a finding confirming the observations from the activity landscapes.

As a next step, we aimed to verify the clinical relevance of our findings and analyzed the effect of different substrate

and cofactor concentrations on the outcome of single dose BH_4-loading tests in individuals carrying different *PAH* genotypes. In clinical routine, the initial phenylalanine concentration at the beginning of the BH_4-loading test is not expected to significantly affect the outcome of the test. In general, only a minimum phenylalanine concentration of 400 μM is considered to be required for reliable test results. Surprisingly, data from BH_4 loading tests available in the BIOPKU database and in the literature (19,33–37,40) does not confirm this view. We learned that patients carrying one of the mutations F39L, I65T or Y414C in either a homozygous or a functional hemizygous state show substantially different responses to the BH_4-loading test in function of the phenylalanine concentration at the beginning of the test. For example, in presence of the mutation I65T, the response may vary from 60% at 500 μM phenylalanine to 0% at 1500 μM blood phenylalanine. This is a new finding that may undermine our trust in current BH_4-loading test protocols. Our results may, for instance, allow for the hypothesis that patients carrying the I65T mutation are at risk to show false negative test results at phenylalanine concentrations >750 μM. In the case of the R261Q mutation, phenylalanine concentrations did not significantly influence test results (Fig. 4B). However, the kind of genotype significantly affected BH_4 responsiveness with carriers of the R261Q mutation in the homozygous state showing a higher response (37.5% decrease in phenylalanine after BH_4 loading) than individuals with the mutation in the functional hemizygous state (6.5% decrease). Further analysis revealed that none of the patients carrying the R261Q mutation in combination with a null mutation met the criterion of BH_4 responsiveness of 30% decrease of phenylalanine concentrations, whereas 12 out of 21 patients with a homozygous genotype did and 9 out of 21 patients did not. Similar observations were recently reported in 27 Turkish PKU patients with a homozygous R261Q genotype and variable clinical phenotypes (11% mild hyperphenylalaninemia, 67% mild PKU, 22% classic PKU), from which only 39.1% were BH_4 responsive (22). Taken together, our results show that the outcome of a BH_4-loading test may much more vary in function of individual test circumstances than previously assumed. Unfortunately, it has to be expected that this is true for a number of mutations and in view of the lifelong consequences for our patients arising from the initial classification of being a responder or not it has to be emphasized that with the knowledge available today results from single-loading tests are not sufficient to determine BH_4 responsiveness in patients with PAH deficiency.

Last, we analyzed the effect of BH_4 dosage on the results of single BH_4-loading tests. Since ~10 years, a dosage of 20 mg/kg body weight is internationally recommended (11,28,29,41). However, in some centers and countries, this recommendation may not be followed. In homozygous R261Q patients, the dosage of BH_4 (10, 20 and 30 mg/kg body weight) did not seem to influence test results (26, 27 and 25% decrease, respectively). In presence of the Y414C mutation, increasing BH_4 dosages increased BH_4 responsiveness in terms of percent decrease of blood phenylalanine after BH_4 application. Interestingly, compound heterozygous patients carrying both the R261Q and the Y414C mutation also showed a dose dependency of response, but to a lower extent than homozygous Y414C patients.

In summary, we developed a rapid PAH enzyme activity assay allowing for a much higher throughput than previous assays and for detailed analysis of a broad range of substrate and cofactor concentrations on PAH enzyme kinetics. This enabled new insights into optimal PAH working range at physiological, pathological and therapeutic conditions. As to enzyme kinetics, two main conclusions can be drawn from our experimental work: phenylalanine concentrations for optimal working range of PAH are lower, whereas BH_4 concentrations for optimal PAH activity are higher than previously assumed. The validity of our observations was substantiated and expanded by the fact that we were able to translate data from the prokaryotic system into the eukaryotic cell culture system and into patient data. Of relevance for the clinical context, we revealed a significant impact of the genotype, substrate concentrations and BH_4 dosage on the assessment of BH_4 responsiveness.

Since the discovery of the pharmacological effect of BH_4 in patients with PAH deficiency (5), the scientific community discusses possible mechanisms of BH_4 responsiveness in PKU. The initial concept was kinetic action, in particular the hypothesis that *PAH* gene mutations lead to decreased affinity of the variant protein to the cofactor, that is overcome by the administration of pharmacological doses of BH_4. Kinetic studies using the recombinant PAH protein revealed that this is true only in rare instances (2,32,42). Subsequent work moved the concept away from kinetic effects toward the view of BH_4 acting as a molecular chaperone by increasing the stability of partially misfolded PAH proteins and by this the effective intracellular concentration of functional PAH enzyme (3,4,12). A deeper view into PAH enzyme kinetics using a technology that allows for the analysis of a broad range of substrate and cofactor concentrations on PAH activity now showed that besides the indubitable chaperone effect, kinetic aspects also have to be taken into account. Thus, we may now put forward the view of both concepts being of relevance for the diagnosis and the treatment of patients with PAH deficiency. The diagnostic loading test with BH_4 or long-term BH_4 treatment has to be seen in the light of the fact that short-term supply of BH_4 can compensate for latent BH_4 deficiency as to optimal catalytic function (kinetic effect), whereas long-term treatment with pharmacological doses of BH_4 increases the stability of PAH and by this the amount of metabolically active enzyme (chaperone effect). In addition, individual mutations may shift the impact of one or the other therapeutic effect.

For daily clinical routine, this underscores the need for even more standardized and at the same time individualized test procedures including detailed documentation of phenylalanine concentrations before the BH_4 load and the awareness that the metabolic status of the patient will influence the outcome of the test. In non-responders with suggestive genotypes, repetition of the loading test at different initial phenylalanine concentrations may help rule out false negative results. Moreover, we suggest to combine short-term BH_4 loading tests (assessment of kinetic effects) and long-term BH_4 treatment

tests (assessment of chaperone effects) with *in vivo* ^{13}C-phenylalanine oxidation tests (assessment of the effect of BH_4 on *in vivo* PAH enzyme activity) (11,43). The test is non-invasive, innocuous, easy to perform and may add important information about an individual's response to the drug at a functional level.

In conclusion, our work pinpoints the importance of genotyping PKU patients even in clinical routine and underscores the need for more personalized testing procedures addressing individual patient characteristics, the metabolic state and the dosage of the test compound to safely identify BH_4 responsiveness in PAH-deficient patients.

MATERIALS AND METHODS

Patients and mutations

Mutations previously identified in BH_4 responsive patients (4,11) were analyzed in terms of the effect of various substrate (L-phenylalanine) and cofactor (BH_4) concentrations on PAH enzyme activity. The mutations mapped to the regulatory domain (F39L, I65T), the catalytic domain (R261Q, P275L, P314S, V388M) or to the dimerization motif of the oligomerization domain (Y414C, Y417H). Forty-six patients homozygous and functional hemizygous for the mutations F39L ($n = 3$), I65T ($n = 5$), R261Q ($n = 28$) and Y414C ($n = 10$) were identified performing a comprehensive literature survey and by extracting data from the BIOPKU database (www.biopku.org). Patients were included in the analysis, when data on a BH_4-loading test using 20 mg/kg body weight and blood phenylalanine concentrations over a period of at least 24 h were available. In addition, the effect of different BH_4 dosages, ranging from 5 to 30 mg/kg body weight, on the course of blood phenylalanine concentrations was analyzed (16,19,34,35,37).

Expression and purification of recombinant PAH proteins

The cDNA of human PAH (EST clone obtained from imaGenes, formerly RZPD, Germany) was cloned into the prokaryotic expression vector pMAL-c2E (New England Biolabs) encoding an N-terminal maltose-binding protein (MBP) tag. *PAH* mutants were constructed by site-directed mutagenesis as previously described (2). Expression plasmids containing the wild-type *PAH* and variants were transformed to *Escherichia coli* DH5α. Expressed proteins were purified by affinity chromatography (MBPTrap, GE Healthcare) followed by size-exclusion chromatography using a HiLoad 26/60 Superdex 200 column (GE Healthcare) on an ÄKTAxpress system (2). Obtained tetramers of the fusion proteins were collected and protein concentrations were determined spectrophotometrically using $\varepsilon_{280\ (1mg/ml)} = 1.63$.

PAH activity assay

Enzyme activity of the recombinantly expressed PAH. The multi-well PAH activity assay and data evaluation were performed as previously described (27) with modifications. L-phenylalanine and 22.35 mM Na HEPES, pH 7.3, were added to all wells of a 96-well plate with different volumes. This resulted in 12 columns of varying L-phenylalanine concentrations (0–4000 μM). A reaction buffer containing 1 mg/ml catalase (Sigma-Aldrich), 10 μM ferrous ammonium sulfate (Sigma-Aldrich) and the tetrameric MBP–PAH fusion protein (0.01 mg/ml) was prepared and injected in all 96 wells. After pre-incubation with L-phenylalanine for 5–20 min, the reaction was initiated by the addition of variable concentrations of BH_4 (6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin, Cayman Chemical) (0–500 μM) stabilized in 100 mM dithiothreitol (DTT; Fluka Chemie). PAH activity was determined at 25°C and 90 s measurement time per well. Using sets of 16 wells and 10 measurement cycles per set, total measurement time for all 96 wells was 22 min. Substrate production was measured by the detection of the increase in L-tyrosine fluorescence intensity, at an excitation wavelength of 274 nm and an emission wavelength of 304 nm, using a fluorescence photometer (FLUOstar OPTIMA, BMG Labtech) and assayed as triplicates. Measured fluorescence intensity signals were corrected by the inner filter effect of BH_4 for every BH_4 concentration added. Enzyme activity measurements were quantified by the measurement of L-tyrosine standards (0–200 μM) before each experiment, and fluorescence intensity was converted to enzyme activity units (nmol L-tyrosine/min × mg protein). Data were analyzed by non-linear regression analysis using the Michaelis–Menten or the Hill kinetic model after comparison of model-fitting using the *F*-test (GraphPad Prism 4.0c) (27). All concentrations mentioned refer to the final concentration in a 202 μl reaction mixture.

Standard PAH activity assay of eukaryotic expressed PAH. PAH enzyme activity was determined as previously described (2,39) with modifications. Twenty microliters of total lysates obtained from cell culture were pre-incubated with 1000 μM L-phenylalanine and 1 mg/ml catalase (Sigma-Aldrich) for 5 min (25°C) in 15 mM Na HEPES pH 7.3, followed by 1 min incubation with 10 μM ferrous ammonium sulfate (Sigma-Aldrich). The reaction was initiated by the addition of 75 μM BH_4 stabilized in 2 mM DTT, carried out for 60 min at 25°C and stopped by acetic acid followed by 10 min incubation at 95°C. All concentrations mentioned refer to the final concentration in a 100 μl reaction mixture. The amount of L-tyrosine production was measured and quantified by HPLC, assayed as duplicates. Three independent experiments were performed.

PAH activity landscapes

The data set of multi-well enzyme activity assayed in a 12 × 8 matrix corresponding to 12 different L-phenylalanine concentrations ranging from 0 to 4000 μM (columns) at 8 BH_4 concentrations ranging from 0 to 500 μM (rows) was loaded into non-linear regression analysis software (GraphPad Prism 4.0c). A non-linear regression analysis was performed for each column of the data matrix in order to extend the sparse data set for BH_4 concentrations from 8 measured to 400 newly calculated values following a substrate inhibition curve. This resulted in a 12 × 400 matrix of activity values. For further calculation of the data and for the creation of landscapes, this data matrix was exported to the free software

package R (www.r-project.org). In order to draw a smooth surface of the landscape, we used the function *interp.loess* from additional R package *tgp* (http://cran.r-project.org/web/packages/tgp/index.html), which interpolates between two data points by using local polynomial regression fitting to find a function between them. This resulted in an increase in data from an originally measured 12×8 (96-well format) over a 12×400 to a 400×400 data set. This grid was then depicted as a smooth landscape plot using the function *image.plot* from package fields (http://cran.r-project.org/web/packages/fields/index.html). To facilitate calculation of landscapes, a script was written accepting comma-separated files and automatically coloring landscapes depending on the measured and interpolated fluorescence intensities.

Stable expression of PAH in HEK293

Stably transfected cells were generated using the Flp-In system (Invitrogen) according to the manufacturer's protocol. The Flp-In-293 cell line was maintained in basic DMEM (PAA Laboratories) supplemented with L-glutamine, high glucose (4.5 g/l), 10% fetal bovine serum (GIBCO), 1% antibiotics (Antibiotic-Antimycotic; PAA) and 100 μg/ml Zeocin (Invitrogen). Cells were stably transfected with pEF5/FRT/V5-DEST cDNA constructs coding for the wild-type, R261Q or Y414C PAH, respectively. Positive clones were selected and maintained in medium containing 150 μg/ml hygromycin B (Invitrogen).

For all further experiments, cells were cultured for 72 h under three different conditions: (i) basic RPMI 1640 medium (91 μM phenylalanine, PAA Laboratories) supplemented with stable glutamine, 10% fetal bovine serum (GIBCO), 1% antibiotics (Antibiotic-Antimycotic; PAA) and 150 μg/ml of hygromycin B, (ii) basic medium (as described above) with 500 μM phenylalanine, and (iii) basic medium with 1200 μM phenylalanine. Additionally, culture conditions were modified by adding 40 or 75 μM BH_4, respectively. Culture medium was changed every 24 h. Cells were harvested and lysed by three freeze-thaw cycles in a Tris–KCl lysis buffer (0.03 M Tris, 0.2 M KCl, pH 7.2) containing protease inhibitors (Roche), followed by 20 min centrifugation at 3000 rcf, 4°C. Recovered supernatants were subsequently used for activity assays.

Statistics

Group mean values were compared by Student's unpaired two-tailed *t*-test. Eukaryotic PAH activities following BH_4 treatment were analyzed by one-way ANOVA and Dunnett's post-test. Statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software).

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